

ATTACHMENT A

Molecular Beacons: Trial of a Fluorescence-Based Solution Hybridization Technique for Ecological Studies with Ruminant Bacteria

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Molecular beacons are fluorescent probes developed for solution rather than membrane hybridization. We have investigated the utility of these probes to study rumen microbial ecology. Two cellulolytic species, *Ruminococcus albus* and *Fibrobacter succinogenes*, were tested. Membrane and solution hybridizations gave similar results in competition experiments with cocultures of *R. albus* 8 and *F. succinogenes* S85.

A new type of fluorescent probe, which is called a molecular beacon, was described recently (13). This probe contains a short complementary sequence of nucleotides attached to the 5' and 3' ends of the probe sequence so that a stem-loop structure forms in solution (Fig. 1). A fluor and a suitable quencher are attached via linkers to the ends of the stem. In the absence of target, the fluor and quencher are held close together by the stem structure and fluorescence is quenched. However, when the single-stranded loop hybridizes with a complementary target sequence, the stem denatures and fluorescence appears because the more stable loop-target hybrid cannot coexist with the internal stem hybrid (13). Because these probes fluoresce strongly only in the presence of their target, they can be used in solution, and removal of unhybridized probe is unnecessary.

The original beacon methodology (13) was developed in a model system with synthetic deoxyoligonucleotide sequences as targets. This approach was modified and applied to ecological studies with ruminant bacteria with 16S rRNA sequences as targets. Molecular beacons were synthesized for *Ruminococcus albus* 8 and *Fibrobacter succinogenes* S85, and results from membrane and solution hybridizations were compared.

Probe design and synthesis. The universal, *R. albus* 8, and *F. succinogenes* S85 probes complementary to 16S rRNA have been described previously (Table 1 [1, 6, 8]). The stem region was designed so that its melting point (T_m) was lower than that of the loop-target region based on base pair number and composition. The GenBank database was searched to ensure that the molecular beacon sequences did not complementarity to any other 16S rRNA sequence.

The starting material was an oligonucleotide with a 5'-methoxytrityl C₆ linker and a 3'-C₇ amino linker (13). These modified oligonucleotides were synthesized at the Oligonucleotide Synthesis Facility, Division of Biological Sciences, Cornell University. Probe construction involved (i) coupling of the 3'-NH₂ with a dabacyl-N-hydroxysuccinimide ester (dabacyl-NHS; quencher) followed by high-performance liquid chromatography (HPLC) purification of the product, (ii) removal of the 5'-SH-protecting group, (iii) reaction of the exposed -SH with iodoacetamidofluorescein followed by HPLC purification.

The methods described by Tyagi and Kramer (13) were followed, with the following modifications. For step i, 20 2.5- μ l aliquots of a saturated solution of dabacyl-NHS in dimethylformamide were added hourly to a stirred solution of the starting oligonucleotide (12 optical density units; 430 μ g) in 300 μ l of 0.1 M NaHCO₃. For step iii, the fluor used was a fluorescein derivative rather than EDANS (5-(2'-aminoethylamino)-1-naphthalenesulfonic acid). This change enhanced the signal strength by a factor of 20 (12). Finally, HPLC purification was done after each of the two chemical couplings rather than only at the end of the synthetic sequence. The overall probe yield in this synthesis (S85 probe) was approximately 10%.

Analytical and preparative HPLC was done with a C₁₈ reverse-phase column (250 by 4.6 mm; pore size, 300 Å; Whatman part no. 4692-3011). The flow rate was 1.0 ml/min at 30°C. A dual-slope linear gradient of 0% (solvent A) to 75% (solvent B) acetonitrile in 0.1 M triethylammonium acetate (pH 6.5) was constructed as follows: 0 to 35 min, 0 to 55% B; 35 to 45 min, 55 to 100% B. UV absorbance at both 260 and 430 nm (step i [probe preparation]) or at 260 and 490 nm (step iii) was monitored. The sensitivity at 260 nm was scaled to 1/10 of that at the longer wavelength.

The synthetic sequence is chemically straightforward, but it is important to purify the intermediate 3'-coupling product by HPLC before continuing with the 5' reaction. The relative mobilities of the starting material and product are depicted in Fig. 2. Addition of the hydrophobic dabacyl group to the 3'-amino linker causes the product to elute later than the starting oligonucleotide. In contrast, replacement of the bulky methoxytrityl group by the more compact acetamidofluorescein causes the product to elute earlier. These same relative shifts were seen for each of the three probes synthesized.

Melting curves. A Perkin-Elmer model 650-15 fluorescence spectrophotometer with a Hitachi digital readout was used with a jacketed cell (type 54FL; NSG Precision, Farmingdale, N.Y.). The cell temperature was controlled with a circulating water bath (Fisher model 9000). Excitation and emission wavelengths were 490 and 535 nm, respectively. The melting curve experiments were conducted by adding the beacon to a final concentration of 1 μ M in 100 mM Tris-Cl buffer (pH 7.2) containing 1 mM MgCl₂ (total volume, 0.7 ml). MgCl₂ was added to stabilize duplex formation between the beacon and its target. The temperature was increased from 27 to 85°C in 5°C increments, and at each increment the fluorescence reading was allowed to stabilize for at least 5 min. The same conditions were used when specificity studies were conducted with the

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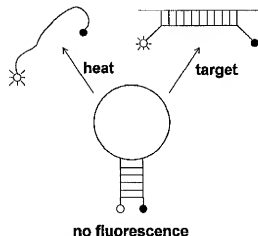


FIG. 1. Dissociation of the base-paired stem structure in the beacon, either by heat or by exposure to a complementary target sequence, releases the quencher and produces a fluorescent signal. O, fluor; ●, quencher. Diagram is (significantly) modified from one by Tyagi and Kramer (13).

oligonucleotide and rRNA targets. The fluorescence of our molecular beacons increased sigmoidally as temperature increased (Fig. 3). Maximum fluorescence indicates the point at which the beacon is in a disordered confirmation (Fig. 1) and the quencher is relatively far from the fluor. The T_m for the stem region (Fig. 3) for each beacon was different and for *F. succinogenes* S85 was the highest (Fig. 3c). This difference in T_m s reflects the differences in the lengths and GC contents of the different stem structures. The *R. albus* curve (Fig. 3b) was atypical and may reflect mispairing of the poly(C)₃poly(G)₃ sequences. In retrospect, this structure was not well designed.

Cultivation of bacteria. *R. albus* 8, *Ruminococcus flavefaciens* FD-1, *Streptococcus bovis* JB1, and *F. succinogenes* S85 were obtained from our culture collection and cultivated anaerobically (2, 3) at 39°C. Rum10 medium (5) was used for the cultivation of *R. albus* 8, *R. flavefaciens* FD-1, *S. bovis* JB1, and *F. succinogenes* S85. Cellobiose (0.4% [wt/vol]) was autoclaved separately and then added as the energy source.

All other bacteria (Table 2) were obtained from Eugene

TABLE 1. Sequences of molecular beacons and oligonucleotide targets

Oligonucleotide ^a	Sequence ^b	FU ^c	Reference
Universal	5'-CCTGCAACGGCGGTGTGTAC GCAGG-3'		6
PM	3'-TGCCGCCACACATG-5'	207 ± 26	
MM1	3'-TGCCGCCACACATG-5'	35.2 ± 6	
MM2	3'-TGCCGCCACACATG-5'	41.6 ± 9	
<i>R. albus</i> 8	5'-CCCCCTATGCGGCTCGTT ATGGGG-3'		8
<i>F. succinogenes</i> S85	5'-GCTGCTGCCCCGAACATC CAAGAGGACGC-3'		1
PM	3'-ACGGGGACTGTATAGTTCT-5'	184 ± 14	
MM3	3'-ACGGGGACGTATAGTTCT-5'	26 ± 11	

^a PM, perfectly matched oligonucleotide target; MMx, single-base mismatched oligonucleotide target, where x indicates different mismatches. Mismatch positions are indicated by the boldface italic font.

^b The underlined region is the inverted repeat that forms the stem, and the nonunderlined region is the probe sequence complementary to 16S rRNA.

^c FU, fluorescence units in hybridization assay.

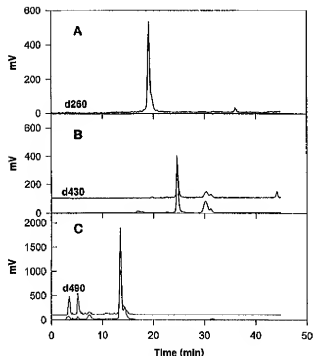


FIG. 2. HPLC profiles from various stages of beacon synthesis. (A) 32-base oligonucleotide containing a probe sequence for *F. succinogenes* and 5'-SH and 3'-NH₂ linkers. This material had been purified by HPLC after synthesis. (B) Product from reaction of this oligonucleotide with dabcyl-NHS ester. The dabcyl amide formed shows an absorbance maximum at 430 nm. Upper and lower traces show signal at 430 and 260 nm, respectively. The total column load in panels A and B was approximately 0.1 optical density unit at 260 nm. (C) Preparative chromatography (ca. 8 optical density units) of the final product after removal of the methoxystyryl-protecting group and reaction with isodansoyl-oligo-fluorescein. The fluorescein residue has an absorption maximum at 490 nm. The lower profile shows the signal at 260 nm.

Madsen (Section of Microbiology, Cornell University). These bacteria were cultured aerobically in nutrient broth with aeration on a rotary shaker.

Specificity of molecular beacons. We observed at least an 80% decrease in fluorescence when our beacons were hybridized to a 5-molar excess of a synthetic target with a mismatch in the middle (Table 1 [MM1 and MM3]) or at the end (Table 1 [MM2]) of the oligonucleotide, indicating that the beacons are highly discriminatory and are destabilized by even a single mismatched base.

rRNA (extracted as described previously [4]) was denatured by heating at 95°C for 5 min. The rRNA solution (3 µg [2.4 pmol]) in a total volume of 50 µl of 100 mM Tris-Cl [pH 7.2], 1 mM MgCl₂ was then transferred to a 39°C water bath, and the mixture was incubated overnight in the presence of the molecular beacon (2 pmol in 50 µl) to allow time for the beacon to hybridize to its target. After the overnight hybridization, the beacon-rRNA hybrid solution was diluted by addition of 0.6 ml of Tris buffer containing 1 mM MgCl₂ and centrifuged (20 min at 4,600 × g) to remove particulate material, and fluorescence at 27°C was measured. Initially, measurements were made while the temperature was increased in 5-degree increments up to 85°C. The differences between the perfect complement and the mismatch were approximately the same for the whole temperature profile. Later measurements were made only at 27°C.

Initial experiments with purified rRNA were confounded by

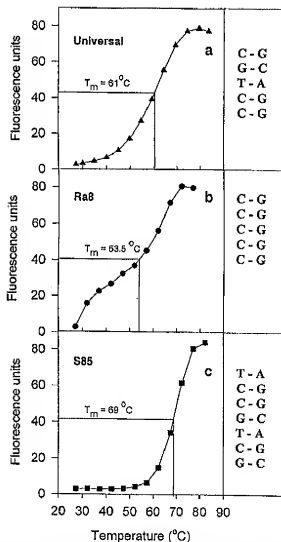


FIG. 3. Melting curves for three beacons showing the increase in fluorescence as a function of temperature. The corresponding stem structures are indicated to the right.

light scattering from rRNA. To optimize wavelengths for rRNA studies, the excitation wavelength was held constant at 490 nm, while the emission wavelength was increased linearly in the range of 510 to 540 nm. At an emission wavelength of 535 nm, the signal from rRNA alone was negligible and accounted for less than 10% of background signal in solution hybridization experiments (data not shown). Light scattering could also be reduced by centrifuging hybridized beacons for at least 20 min. When standard curves were constructed, the total amount of rRNA in each assay had to be equalized by adding nontarget rRNA to correct for background. When chemical denaturants such as glutaraldehyde and formamide were used, both the rRNA and the beacon were denatured; however, unlike membrane hybridization, the chemical denaturant cannot be easily removed and tends to generate high background fluorescence. Attempts to dilute out the denaturant were not successful.

Beacons were highly specific and did not hybridize with nontarget rRNAs (Table 2). Computer-based searches (Gen-

TABLE 2. Specificities of molecular beacons tested against a phylogenetically diverse group of bacteria

Bacterium (ATCC no.)	Molecular beacon		
	Universal	<i>R. albus</i>	<i>F. succinogenes</i>
<i>Bacillus cereus</i> (14579)	+	—	—
<i>Bacillus megaterium</i> (14581)	+	—	—
<i>Bacillus subtilis</i> (6051)	+	—	—
<i>Escherichia coli</i> K-12	+	—	—
<i>Fibrobacter succinogenes</i> (19169)	+	—	+
<i>Micrococcus luteus</i> (4698)	+	—	—
<i>Pseudomonas cepacia</i> (25416)	+	—	—
<i>Pseudomonas fluorescens</i> (13525)	+	—	—
<i>Ruminococcus albus</i> (27211)	+	+	—
<i>Ruminococcus flavefaciens</i> (19208)	+	—	—
<i>Streptococcus bovis</i> JB1	+	—	—

Bank) indicated no homology of the beacons, including the stem structure, with any other 16S rRNA. It should be noted that our probes had previously been tested for specificity (Table 1) (1, 6, 8), and it was not expected that cross-reactivity would be a problem.

Hybridization experiments. When *Escherichia coli* K-12 and *F. succinogenes* S85 rRNA were mixed in known ratios, the relationship between observed and predicted fluorescence values for *F. succinogenes* with an S85-specific probe was linear (Fig. 4). The amount of S85 rRNA was calculated from a calibration curve constructed by measuring the fluorescent signal from a fixed amount of probe and increasing amounts of purified S85 rRNA. The total rRNA was determined in a similar manner by using the universal beacon. *F. succinogenes* S85 16S rRNA was expressed as a percentage of total 16S rRNA. Input and measured fractions of S85 rRNA were linearly related ($r^2 = 0.90$), and the slope did not differ from unity.

The accuracy of solution hybridization was evaluated by comparison to membrane hybridization (Fig. 5). Methods for the extraction of rRNA and slot blot hybridization with the

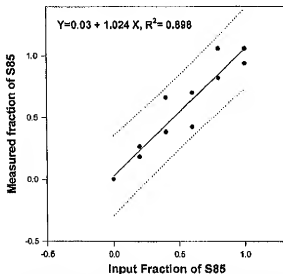


FIG. 4. Probing of known mixtures of purified *E. coli* K-12 and *F. succinogenes* S85 rRNA with an S85-directed beacon. Solid regression line, together with 95% confidence intervals (dotted lines), is shown. Replicate values were plotted separately.

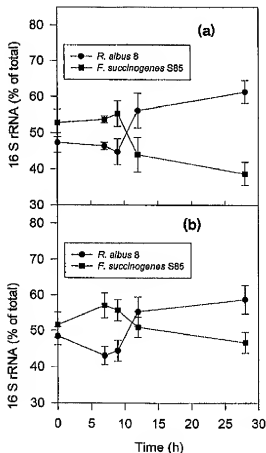


FIG. 5. Comparison of membrane (a) and solution (b) hybridizations in competitive studies between *F. succinogenes* S85 and *R. albus* 8.

Genius System 7 (Boehringer Mannheim) have been described previously (4). All solutions were autoclaved for 30 min and then treated with 0.01% diethylpyrocarbonate. Competition experiments between *R. albus* 8 and *F. succinogenes* S85 were conducted in 100-ml serum bottles containing Rum10 medium with sterile anaerobic cellobiose (0.4% [wt/vol]) added to each bottle at the beginning of each experiment. Healthy overnight cultures of *R. albus* 8 and *F. succinogenes* S85 were inoculated into each serum bottle, and the bottles were incubated at 39°C. Twenty-milliliter samples were taken at 0, 7, 9, 12, and 28 h of incubation and were immediately frozen at -20°C. When *R. albus* 8 and *F. succinogenes* S85 were grown in coculture, similar results were obtained for membrane (Fig. 5a) and solution (Fig. 5b) hybridizations. *F. succinogenes* S85 was the predominant bacterium until 12 h, but thereafter *R. albus* 8 became dominant. Good regression fits were obtained when solution and membrane hybridization values were compared (Fig. 6). However, the fit was better for *R. albus* 8 (Fig. 6a) than that for *F. succinogenes* S85 (Fig. 6b).

rRNA-based identification allows direct detection of bacteria in an ecosystem without cultivation because oligonucleotide probes to signature regions of target rRNA can be designed (11). Detection is usually based on immobilization of the target rRNA on a solid support (nylon), hybridization with the probe, and finally removal of unhybridized probe (10). Unhybridized probe must be removed in most solution hybridization systems

in which the target is not immobilized on a solid support. The stem-loop structure of the molecular beacons described by Tyagi and Kramer (13) makes removal of unhybridized probe unnecessary and is an inherent property of the competition between the stem and loop features of the beacon.

Intramolecular competition between the internally base-paired stem and pairing of the loop with an external target can be used to control stringency. To take full advantage of this potential stringency, one may design the stem sequence so that the difference in T_m between the base-paired stem and loop-target hybrid is relatively small (about 10°C). The T_m for the loop can be calculated by standard equations (10, 15). However, these equations do not suffice for the stem because of the small number of bases and because intramolecular bonding removes concentration-dependent factors. One way to address this problem is to plot the standard free-energy values for secondary-structure loops in RNA with RNAdraw V1.0 (7) against the observed probe T_m (Fig. 7). One then may estimate the T_m for each stem from this plot and choose an appropriate stem structure based on T_m . We assume that only small energy differences exist between RNA-RNA and RNA-DNA duplexes when short (<10-bp) sequences are involved. As more probes are synthesized, this correlation among stem sequence, stem stabilization energy, and stem T_m will become more useful for stem design. We designed the three probes described in this paper without a full appreciation of the importance of the stem sequence. The probes described were not optimized by using

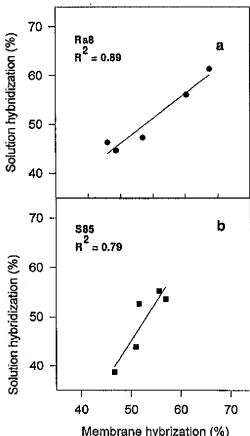


FIG. 6. Regression of solution hybridization values on membrane hybridization values with *R. albus* 8 (a) and *F. succinogenes* S85 (b).

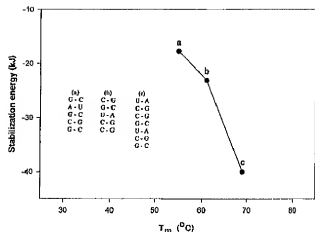


FIG. 7. Regression of stabilization energy values resulting from base pairing as calculated by RNAdraw (7) against measured T_m values. The following stem structures are also shown: molecular beacon A (13) (a), universal probe (this paper) (b), and *F. succinogenes* S85 probe (this paper) (c). Data for the *R. albus* 8 probe were not included because of this probe's unusual stem structure and biphasic melting curve.

RNAdraw. Nevertheless, the probes performed well and it appears that some latitude in stem design is permissible.

One advantage of using molecular beacons to study microbial ecology is that the same probes developed against rRNA by using membrane support formats can be modified to produce the stem-loop beacon structure. However, secondary structure in rRNA may limit the access of a beacon to its target. Membrane hybridization circumvents this problem by holding the rRNA in a denatured state on a solid surface. If enough sequence information is available to estimate rRNA secondary structure, then beacons can be targeted at less highly structured regions. Without this information, as in the present case, rigorous optimization with both target and nontarget rRNAs is essential.

At least three processes which are directly influenced by rRNA secondary structure occur simultaneously in the solution hybridization assay: (i) refolding of rRNA after denaturation, (ii) refolding of the beacon, and (iii) interaction of the beacon with its target rRNA. We were able to optimize this process by heating the rRNA target in the presence of the beacon and then allowing the mixture to cool slowly overnight. In contrast, the much simpler oligonucleotide targets hybridized rapidly at room temperature.

The specificities of the beacons were high when they were tested against mismatched oligonucleotide targets. Tyagi and Kramer (13) reported that there was no fluorescence in the presence of a 1-base mismatched oligonucleotide target; however, we obtained some fluorescence with 1-base mismatches. It should be noted that Tyagi and Kramer (13) used a highly idealized model system, in which the properties of the beacons could be optimized. Our beacons performed almost as well

with a biologically realistic target. They did not hybridize to nontarget rRNA and were highly specific when tested against a phylogenetically diverse group of bacteria (Table 2).

Competition experiments between *R. albus* 8 and *F. succinogenes* S85 showed that membrane and solution hybridizations gave similar results (Fig. 5a and b) and good regression fits (Fig. 6a and b) compared with one another. Odenyo et al. (8) found that *F. succinogenes* S85 dominated a coculture of *F. succinogenes* S85 and *R. albus* 8 until 12 h but that *R. albus* 8 predominated after 12 h. Our longer incubations showed that from 12 to 28 h, the level of *F. succinogenes* S85 declined. *R. albus* 8 and *F. succinogenes* S85 have similar growth rates on cellobiose (9); however, *F. succinogenes* S85 is a rapidly lysing bacterium (14), and lysis probably accounts for the decline in *F. succinogenes* S85 levels in the later stages of competitive growth (Fig. 5a and b).

Molecular beacons provide an additional, powerful tool for the study of microbial ecology. One of the big advantages of solution hybridization is that quantitative results can be obtained within 12 h, while membrane hybridization can take 3 to 4 days.

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